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Physiological and biochemical characterization of *Xanthomonas axonopodis* PV. *citri*: A gram negative bacterium causing citrus canker

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Abstract
Citrus canker caused by *Xanthomonas axonopodis* pv. *citri* is one of the most important disease of citrus. In the present study, ten isolates of *X. axonopodis pv. citri* were isolates from different regions of Vidharbha. A several biochemical test were conducted to differentiate the disease. All isolates were found positive for starch hydrolysis, KOH test, catalase test, H_2S production, gelatin liquefaction, indole production, acid and gas production tests to performed to characterized the *X. axonopodis* pv. *citri* bacteria. The result of all bio-chemical test confirmed that, *Xanthomonas* is gram negative bacterium.

Keywords: soybean, pathogens seed borne mycoflora, standard blotter paper

Introduction
Citrus, including important crops like oranges, lemons, grapefruit, pomelo and lime. It grown in the world with tropical or subtropical climates. It is used as best source of vitamin C, sugars, amino acids and other nutrients. It is one of the important fruit crops of the world. It occupies an important place in the wealth and economy of India as third largest fruit industry after mango and banana. In India, citrus is grown in an area of 1,024 thousand hectares with a total production of 11,581 thousand MT (Anonymous, 2016) [2]. In Maharashtra state, citrus is grown on 287.6 thousand hectare with production of about 1725.1 MT fruits annually (Anonymous, 2016) [2]. The most important commercial citrus cultivars in India are the mandarin (*Citrus reticulata*) followed by sweet orange (*Citrus sinensis*) and acid lime (*Citrus aurantifolia*). Citrus canker is one of the most destructive and predominant disease on acid lime in Vidarbha region of Maharashtra. Citrus bacterial canker (CBC), caused by *Xanthomonas citri* subsp. *citri* (Schaad *et al.,* 2006) [16] is one of the most devastating diseases throughout the world that affects many kind of commercial citrus varieties. The origin of CBC is known but thought to have originated from south-east Asia or India and then widely distributed around the world (Civerolo, 1984 and Vernière *et al.,* 1991) [3]. According to, Fawcett and Jenkins (1933) [6] the origin of citrus canker is either from India, Java or some other region of Asia. It was first identified in Florida (USA) in 1915 and in India was reported from Punjab in 1942. The main symptoms of CBC are hyperplasia type lesions on leaves, fruit and stems. The severe infections causes leaf abscission, twig dieback and premature fruit drop (Gottwald *et al.,* 2002) [8]. The bacterium was first named as *Pseudomonas citri* (Hasse, 1915) [9]. In 1939 it was classified as genus *Xanthomonas* spp. *(X. citri),* then reclassified in 1980 (Dye *et al.,* 1980) [5] as *Xanthomonas campestris* pv. *citri* due to inadequate phenotypic data (Young *et al.,* 1978) [18].

Material and Methods
Isolation and purification of *Xanthomonas axonopodis* pv. *citri* (*Xac*)
The Infected leaves of citrus canker were collected from declining orchard and cut into small pieces and crush it into a drop of distilled water. Streak that smear on NA medium plate with the help of wire loop and incubated for 24 hrs. at 30^0 C. Take a loop full culture and streak on another media plate for pure culture.

Pathogenicity test
Pot culture (seedling inoculation) technique
The ten seedlings of acid lime were used for inoculation of each isolate separately. Inoculation

was done by smearing the bacterial culture on leaves at 10 injury points made by pin prick method. The plants were maintained under humid condition. The observations were recorded on the basis of number of pricks made and number of spots exhibited diseased symptoms. Uninoculated injured plants treated with sterilized water served as control.

Detached leaf technique

Detached leaf technique was followed to prove the pathogenicity. Middle age leaves of acid lime were selected and detached from the plants. They were washed with tap water, swabbed with 70% ethanol and allowed to dry. Then injuries were made at several points by pricking with sterilized needle and smeared on both sides with culture soaked sterilized cotton swab and dipped in 2 % sucrose solution. The leaves were kept in plates which lined with sterilized moist filter paper to maintained humidity and incubated at 30^0C.

Physiological characters

Temperature requirement

The study was conducted to know the optimum temperature requirement for the growth of *Xanthomonas axonopodis* pv. *citri* using nutrient agar broth as a basal medium. A loop full of 48 hours old bacterial culture was mixed in 100 ml of broth in 250 ml flasks. Then inoculated flasks were incubated at different temperature level viz., 5, 10, 15, 20, 25, 30, 35 and 40^0C respectively for 72 hours. Observations were recorded for the growth of bacterial colonies in the inoculated flasks kept at specific temperature levels. The growths of isolates were studied turbidometrically after 72 hours using spectrophotometer at 600 nm.

pH requirement

Effect of hydrogen ion concentration of the growth of *Xanthomonas axonopodis* pv. *citri* was studied by adjusting the pH of the medium (NA broth) to various levels viz., 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 using appropriate phosphate buffer. A loop full of 72 hours old bacterial culture was mixed in 100 ml broth in 250 ml flask. Inoculated flasks were incubated at 28 ± 2^0C temperature for 72 hours. After the incubation period, observations were recorded for the growth of the bacterium in the media having different pH level. The growths of isolates were studied turbidometrically after 72 hours using spectrophotometer at 600 nm.

Biochemical studies

Biochemical tests *viz*., Gram staining, catalase test, KOH test, starch hydrolysis, gelatin liquefaction, H_2S production, indole production, acid and gas production etc. were carried out for biochemical confirmation of *Xanthomonas axonopodis* pv. *citri* according to Aneja, 2003 [1].

Gram reaction

The drop of suspension was smeared over the slide and air dried. Then dried smear was fixed by passing the slide 3-4 times rapidly over the flame. The smear was flooded with crystal violet for 30 seconds and washed in the tap water. Then the smear was immersed in potassium iodide/ Lugol's iodine solution for 30 seconds, washed in tap water then decolorized with 95% alcohol and rinsed with water. Counter stained with saffranin for 10 seconds, again washed with tap water and air dried. Drop of cedar wood oil was placed on the slide and examined the smear under oil immersion lens.

Catalase test

A loop full of 24 hours old culture of the test bacterium was placed on the clean glass slide and to this a drop of 3% hydrogen peroxide (H_2O_2) was mixed and allowed to react for few minutes and observed for the production of gas bubbles.

KOH test

Two drop of 3% potassium hydroxide (KOH) was placed on a glass slide. A loop full culture was picked up from the medium with help of inoculating needle and mixed with KOH drop for 10 seconds and raised the needle for 0.5 to 2 cm to form thread which was treated as positive test.

Starch hydrolysis

Bacterial culture was inoculated on starch agar plates (nutrient agar + 0.2% soluble starch) and incubated for 7 days. After incubation the plates were flooded with Lugol's iodine solution (test reagent). Presence of starch hydrolysis indicated by the appearance of golden yellow zone around the inoculated colony. The yellowish zone indicated that the starch was partially hydrolyzed to dextrin.

Indole production

The medium (10 g of tryptophan in one liter of distilled water) was distributed in test tubes and autoclaved. The culture of tested isolates were inoculated in these test tubes under aseptic condition and incubated for 48 hrs. After 48 hours of inoculation, Kovac's reagent (1ml) was added in each incubated test tube. The tubes were allowed to stand to permit the reagent to come to top. Development of cherry (deep) red color in the top layer of the tube is positive test for indole production. Absence of red coloration is indole negative test.

Gelatin liquefaction

Bacterial culture were inoculated through stab of a nutrient gelatin tube (nutrient broth + 1.5% gelatin) and incubated for 7 days, uninoculated tubes serve as a control and observed for liquefaction. Deep gelatin incubated tubes that remain liquefied produced gelatinase and showed positive test for gelatin hydrolysis and those tubes that remain solid demonstrate negative reaction for gelatin hydrolysis.

Acid and gas production from carbohydrates

Dextrose broth (nutrient broth + 0.5% dextrose) was prepared with the test reagent (20 ml broth + 0.2 ml bromo cresol purple). Ten ml dextrose broth distributed in each test tube and placed one durham tube in inverted position in each test tube. After sterilization of test tube, inoculated with *Xanthomonas axonopodis* pv. *citri* isolates and incubated for 3 days at room temperature. If the color of indicator changes blue to yellow it indicates the formation of acid and gas by its accumulation in durham tube.

H_2S gas production

Ten ml of SIM agar media (beef extract-3 g, peptone-30 g, ferrous ammonium sulphate-0.2 g, sodium thiosulphate 0.025 g and distilled water) was poured in each test tubes and autoclave at 15 psi for 15 min. A loop full culture of *Xanthomonas axonopodis* pv. *citri* was inoculate by means of stab inoculation in each test tube, then lead acetate paper was inserted in these tubes and hold by the plugs above the culture without touching the medium. The tubes were inoculated at 30^0C for three days. If filter paper strips turns black, it indicated the positive test for H_2S production.

Result and Discussion

Pathogenicity by detached leaf technique

All the Xac isolates were reisolated from inoculated leaves and reidentified by morphological characters. Isolate Xac-10 (Akola) gave 3 mm water soaked lesions surrounded by yellow halo zone. No symptoms were observed in control plate inoculated with sterilized water by pin prick method. Pathogenic ability of ten different isolates of Xac were confirmed and found that isolate Xac-10 showed highly pathogenic to initiate water soaked lesion and fully developed symptoms after 10 days under *in vitro* and 15 days under *in vivo* condition. While Xac-2, Xac-3, Xac-5, Xac-6 and Xac-7 were not found any canker lesions however, Xac-1, Xac-4, Xac-8 and Xac-9 were found moderate lesions of canker under *in vitro* condition. Whereas Xac-2, Xac-3, Xac-5, Xac-6 and Xac-7 were found weak canker lesions and Xac-1, Xac-4, Xac-8, and Xac-9 were found moderate canker lesions on leaves under *in vivo* condition (Table-1). Katkar *et al*. (2016) [11] categorized the fifteen isolates of Xac on the basis of symptoms development on leaves and days taken for appearance of the symptoms as no canker (-), weak canker (+), moderate canker (++) and strong canker (+++) as presented in table 1.

Table 1: Pathogenic ability of *Xanthomonas axonopodis* pv. *citri* on acid lime leaves *in vitro* and *in vivo* experiment.

Sr. No.	Isolates	No. of days required for development of symptoms		Lesion in mm		Symptoms	
		in vitro	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*
1	Xac-1	13	16	1.2	1.3	++	++
2	Xac-2	15	24	0	1.0	-	+
3	Xac-3	15	25	0	0.8	-	+
4	Xac-4	12	18	0.7	1.2	+	++
5	Xac-5	15	25	0	0.6	-	+
6	Xac-6	15	24	0	0.8	-	+
7	Xac-7	15	25	0	0.8	-	+
8	Xac-8	13	18	1	1.3	+	++
9	Xac-9	12	18	1.3	1.6	++	++
10	Xac-10	10	15	3	2.5	+++	+++

Note- (-) no canker, (++) moderate canker, (*in vitro*- leaf detached technique)
(+) weak canker, (+++) strong canker. (*in vivo* - inoculation on seedling)

Table 2: Effect of temperature on growth of *X. axonopodis* pv. *citri*.

Temp.	Optical density (72 h) at 600nm										Mean
	Xac-1	Xac-2	Xac-3	Xac-4	Xac-5	Xac-6	Xac-7	Xac-8	Xac-9	Xac-10	
5^0C	0.08	0.08	0.06	0.05	0.05	0.04	0.04	0.07	0.07	0.9	0.06
10^0C	0.16	0.19	0.21	0.18	0.20	0.17	0.18	0.23	0.17	0.24	0.19
15^0C	0.31	0.30	0.27	0.25	0.25	0.23	0.25	0.30	0.30	0.33	0.27
20^0C	0.37	0.36	0.33	0.31	0.32	0.30	0.31	0.36	0.35	0.41	0.34
25^0C	0.66	0.64	0.60	0.60	0.59	0.65	0.60	0.64	0.63	0.70	0.63
30^0C	0.90	0.90	0.86	0.88	0.90	0.84	0.92	0.90	0.94	0.96	0.90
35^0C	0.50	0.48	0.43	0.43	0.44	0.40	0.41	0.50	0.51	0.60	0.47
40^0C	0.13	0.15	0.12	0.10	0.10	0.08	0.08	0.11	0.12	0.18	0.11

Table 3: Effect of pH level on growth of *X. axonopodis* pv. *citri*.

pH	Optical density (72 h) at 600nm										Mean
	Xac-1	Xac-2	Xac-3	Xac-4	Xac-5	Xac-6	Xac-7	Xac-8	Xac-9	Xac-10	
3.0	0.16	0.15	0.13	0.11	0.11	0.09	0.11	0.12	0.14	0.20	0.13
4.0	0.27	0.24	0.20	0.20	0.18	0.16	0.19	0.22	0.25	0.31	0.22
5.0	0.43	0.40	0.38	0.38	0.36	0.35	0.39	0.42	0.45	0.49	0.40
6.0	0.70	0.68	0.63	0.64	0.61	0.55	0.59	0.68	0.70	0.78	0.59
7.0	0.87	0.84	0.80	0.80	0.78	0.75	0.81	0.83	0.85	0.93	0.82
8.0	0.58	0.56	0.54	0.50	0.50	0.46	0.51	0.54	0.58	0.65	0.54
9.0	0.21	0.18	0.18	0.19	0.17	0.19	0.17	0.20	0.18	0.25	0.19

Physiological characters

Effect of temperature regimes on growth of *Xanthomonas axonopodis* pv. *citri*

The data in table-2 indicates the effect of various temperature level on the growth of *Xanthomonas axonopodis* pv. *citri* was recorded by measuring their optical density value. The maximum optical density value of bacterial growth was at 30^0C with an optical density value of 0.90. The next favorable temperature for the growth of bacterium was 25^0C with OD value of 0.63, followed by 35^0C with OD value of 0.47, 20^0C with OD value 0.34, 15^0C with OD value 0.27, 10^0C with OD value 0.19. At 5 and 40^0C there was the least growth of the bacterium with OD value of 0.06 and 0.11 respectively.

Effect of pH on growth of *Xanthomonas axonopodis* pv. *citri*

The data presented in table-3 revealed that maximum growth of the bacterium was recorded at pH level 7.0 with OD value of 0.82 followed by pH 6 with OD value 0.59, pH 8 with OD 0.54, pH 5 with OD value 0.40. The least growth of pathogen was recorded at pH level of 3.0, 4.0 and 9.0 with OD value of 0.13, 0.22 and 0.19 respectively which are significantly at par. Kiran Kumar (2007) [12] observed that, the optimum temperature for the growth of bacterium was found to be 28-32^0C and minimum and maximum temperature were 10 and 40^0C respectively. The optimum pH required for the growth was 7.0-7.2, while, minimum and maximum pH was 5 and 9 respectively. Similar result was also reported earlier by

Hingorani and Mehata (1952) [10], Manjula (2002) [13], Giri (2009) [7] and Yenjerappa (2009) [17].

Bio-chemical test

Bacterium *Xanthomonas axonopodis* pv. *citri* is rod shaped, Gram negative, circular pale yellow colonies on nutrient agar. As earlier reported by Patel (1950) [14]. He observed bacterial colonies of *X. malvacearum* as flat glistering pale yellow on nutrient agar also identified the organism is short rod with rounded ends, Gram negative. Manjula (2002) [13] reported, seven isolates of the pomegranate bacterium were small rods, appeared singly, Gram negative. Gottwald *et al.* (2002) [8] reported *Xanthomonas axonopodis* is a rod shaped, Gram negative bacterium. Das (2003) [4] reported that the bacteria are rod shaped measuring 1.5-2.0 x 0.50.75 µm, Gram negative. Biochemical tests *viz*., KOH, catalase, starch hydrolysis, gelatein liquefaction, acid and gas production, indole production confirms the bacterial pathogen *Xanthomonas axonopodis* pv. *citri* as a Gram negative bacterium. The cultured showed variation among the isolates of *Xanthomonas axonopodis* pv. *citri*. Similar variation among the isolates has been earlier noted by Raut (1990) [15] studied 15 isolates of *Xanthomonas axonopodis* pv. *mangiferae indicae* for different physiological and biochemical properties *viz*. H_2S production, action on carbohydrates, gelatin test, KOH test etc.

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